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Original communication

Demonstration of ethyl glucuronide in dental tissue samples by liquid chromatography/electro-spray tandem mass spectrometry

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ABSTRACT

Introduction: Ethyl glucuronide (EtG) has been studied in various tissues and body fluid for determination of alcohol intake. However, no study, dealing with EtG analysis in dental tissue, was performed so far. In this study, we aimed to demonstrate EtG levels in dental tissue.
Materials and methods: Michigan Alcohol Screening Test (MAST) was performed to 29 participants. Following the test, cases were divided into three groups as non-hazardous alcohol users, alcohol abusers and 6 controls who verbally declared that they were abstainers. A total of 29 tooth specimens, obtained from participants, was included in the study. These specimens were analyzed using LC/MS/MS.
Results: All of the participants included in the study were male. According to the MAST outcomes 14 of the participants were non-hazardous alcohol users, and 9 were alcohol abusers, while 6 patients verbally declared that they were abstainers. Dental tissue analyses revealed EtG levels ranging between EtG < LOD and 23.39 pg/mg. EtG levels were observed to be <LOD in dental specimens of 6 abstainer cases. A significant correlation was found between EtG levels measured in the dental tissues and MAST outcomes on the statistical analyses ($r = 0.914$).
Conclusion: The findings of the present study demonstrated that dental tissue can be used for detection of alcohol intake, using LC/MS/MS.

1. Introduction

Alcohol use continues to rise throughout the world.[1] Thereby, alcohol related issues are still of high importance in daily forensic practice. Alcohol is metabolized following intake in living ones, while microbial activity and fermentation of glucose after death may lead to postmortem production of alcohol under some circumstances in the corpses, which might cause serious problems during evaluation of forensic cases.[2–5] Determination of levels of consumed alcohol is unreliable in some cases because of alcohol levels possibly produced by microbial activity. And detecting an accurate level of ethanol became impossible in such cases. Therefore, ethyl glucuronide (EtG), a minor metabolite of ethyl alcohol, is used for this purpose.[6–8] By a number of researchers, EtG has been previously demonstrated in various tissues and body fluid for revealing alcohol intake.[6,9–12] However, to date, no study dealing with EtG analysis in dental tissue was performed.

Dental tissue is one of the specimens used in forensic medical applications and analyses.[13] Because it is a live tissue that give response to physiological and pathological alterations with its micro-circulation.[14–16] Dental tissue is of high importance due to its durable structure in case of the deterioration of the body integrity caused by diseases, postmortem changes and severe traumatic events.[13,17]

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In this study, to the best of our knowledge as the first study in this field we aimed to demonstrate EtG levels in dental tissue using LC/MS/MS method and to reveal its correlation with alcohol intake behavior.

2. Materials and methods

2.1. Ethics statement and subject

The tooth specimens used in this study were obtained from the patients presented to Dental Clinic of Mustafa Kemal University, Medical Faculty for examination. Extracted teeth of the patients in whom the decision was made for tooth extraction were taken after informing them about the study. Informed consent was given by all participants. Michigan Alcohol Screening Test (MAST) was performed to 29 participants. Following the test, cases were divided into three groups as non-hazardous alcohol users, alcohol abusers and 6 controls who verbally declared that they were abstainers. Twenty-nine tooth specimens obtained from these patients were included in the scope of the study. The specimens were preserved in clean Eppendorf tubes and consequently analyzed with LC/MS/MS method. The protocol of this study was approved by the Mustafa Kemal University Ethics Committee.

2.2. Chemicals, reagents and materials

EtG and deuterium-labeled EtG-d5 standards (internal standard) were obtained from Medichem (Stuttgart, Germany). All solvents were hypergraded for LC–MS LiChrosolv and purchased from Merck KGaA (Darmstadt, Germany). Deionized water was obtained from the Milli-Q (Millipore, Bedford, USA) water purification system.

Stock solutions of EtG (10 μg/ml) and d_5-EtG (2.5 μg/ml) were prepared in methanol and they were stored at −20 °C. Working standard solutions used for calibration and quality control samples were prepared by 2, 5, 10, 20, 50, 100, 200, 1000 and 2000 ng/ml. All working solutions were stored in a refrigerator when not in use.

2.3. Specimen preparation

Extracted teeth were cut from the root part containing dentin. After the addition of 5–6 steel ball bearings (2 mm diameter) to each vial, the capped vials were placed in a Mini-Bead-Beater-8, a high energy cell disrupter (BioSpec Products, Bartlesville, OK, USA) for approximately 3 min or until powdered.

The powderized specimen of 50 mg was weighed with a sensitive scale and placed in a tube. A mixture of 50% acetonitrile/50% water was added on it, and this mixture was kept in an ultrasonic bath at 25 °C for 2 h. Then, internal standard of 50 μl was added on it and mixed with vortex. Then it centrifuged at 4000 rpm for 10 min. 2 ml was extracted from the upper part and placed in the autosampler vials. The specimens were separately subjected to extraction with mixtures of water/acetonitrile/methanol, acetonitrile/water of 80% and acetonitrile/water of 50% and the best result was obtained with the mixture of 50% acetonitrile/50% water.

2.4. LC–MS/MS conditions

The specimens were analyzed using an Agilent Technologies 1200 system that consisted of a G1367C autosampler, a G1379B degasser, G1312B binary pump. Separation was achieved using two Zorbax Hilic Plus (4.6 × 100 mm, 3.5 micron particle size) serial connected column. Reverse–reverse chromatographic technique was used. The column was held at 25 °C in a G1316B Thermostatted Column Compartment (Wilmington, DE, USA). The solvent system was a gradient that consisted of A (1 mM NH4Ac) and B (acetonitrile), using a flow rate of 0.8 mL/min. The solvent program held B at 65% from 0.0 min to 2.2 min. Solvent B was decreased to 20% between 2.3 and 9.5 min. Solvent B was increased to 20% at 5.1 min and held at 65% until 10.0 min. The detector was Agilent Technologies 6460 Triple Quad LC/MS System using electro-spray ionization (ESI) in the negative mode (Wilmington, DE, USA). The capillary voltage was set at 4000 V, the nozzle voltage set at 0 V and the desolvation gas (nitrogen) was heated to 350 °C with a flow of 11 l/min. Nebulazator pressure: 50 psi. The sheath gas (nitrogen) was heated to 350 °C and delivered at 11 l/min.

The internal standard (ETG-d5) was monitored using the m/z 226.0 > 75.0 (quantification ion) transition and the m/z 226.0 > 85.0 (qualifying ion) transition. The m/z 221.0 > 75.0 (quantification ion) and m/z 221.0 > 85.0 (qualifying ion) transitions were used to monitor ETG. All three transitions used a fragmentor voltage of 100 V and collision energy of 12 V. All data were processed using MassHunter B.04.01 (Wilmington, DE, USA).

Start	RT	End	Height	Area	Signal To Noise
1.986	2.045	2.13	14	62	12.4

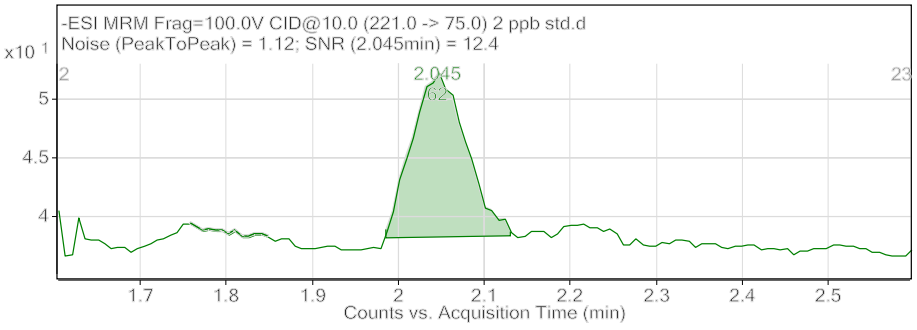


Fig. 1. Integration peak list.

Table 1
EtG spikes of the same sample of 5, 20 and 50 pg/mg.

50 ACN ext.d	21,450[a]
50 ACN ext + 5 pg/mg EtG spiked.d	74,771
50 ACN ext + 20 pg/mg EtG spiked.d	221,641
50 ACN ext + 50 pg/mg EtG spiked.d	511,913

[a] pg/mg.

Table 2
The levels of EtG in teeth (mean ± SEM).

Group	EtG[a]	*p*	MAST[b] points	*p*
No alcohol	<LOD[c]		0.00	
Light user	6.20 ± 2.32	0.000	3.57 ± 0.64	0.000
Heavy user	21.55 ± 1.07		8.33 ± 0.70	

[a] pg/mg.
[b] MAST point 0–4: non-hazardous alcohol user, 5–9: alcohol abuser, >10: alcohol addiction.
[c] LOD: Limit of Detection.

2.5. Identification criteria

The identification criteria used for this procedure included four components: retention time, relative ion intensity signal to noise and baseline resolution.

2.6. Validation

Analytical quality assurance study of the method was performed with definitions of the parameters, including selectivity, linearity, recovery, limit of detection (LOD) and limit of quantitation (LOQ).

2.6.1. Selectivity

Selectivity (specificity) is an indicator of the extent of interference for a particular analyte with the other components in a mixture. Within the selectivity studies, 5 blind specimens were analyzed. These specimens were subjected to the Specimen preparation process and analyzed. No any peak was found to lead to interference.

2.6.2. Linearity

The linearity is the ability of analytical procedure to produce test results, which are proportional to the concentration (amount) of analyte in samples within a given concentration range. In order to define the linearity, EtG standard solutions were prepared in a concentration of 10 μg/ml, and the linear range was defined as 2–2000 pg/mg. To assess linearity, a calibration curve was prepared in replicate ($n = 3$) and analyzed. The acceptance criteria were: a correlation coefficient (r) and determination coefficient (r^2) >0.99, and a precision and accuracy, for the back calculated concentrations of the calibration points, within ±15%.

2.6.3. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection in an analytical procedure is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated as an exact value. It is expressed as a concentration at a specified signal:noise ratio (SNR), usually as 3:1. The limit of quantitation is the lowest amount of the analyte in the sample that can be quantitatively determined with defined precision under the stated experimental conditions. It is expressed as a concentration at a specified signal:noise ratio (SNR), usually as 10. For the method used in this study, limit of detection was found as 0.48 pg/mg and limit of quantitation as 1.61 pg/mg. The values obtained were confirmed by analyzing of the samples with addition in the specified concentrations (Fig. 1).

2.6.4. Accuracy

The accuracy can be determined by extracting a relevant certified reference material (CRM) and calculating the percentage recoveries relative to the certified values. Because we had not certified reference materials of tooth extracts samples, accuracy examinations were made with calculating the percentage recoveries. Recovery is a measure of the retaking rate of the sought analyte added into the sample. For this purpose, standard EtG of 5, 20 and 50 pg/mg was added to the samples and re-analyzed (Table 1).

2.7. Statistical analysis

Statistical evaluations were performed using SPSS for Windows 15.0 (Statistical Package for Social Sciences) software package was used. Continuous variables were analyzed by Kolmogorov–Smirnov test in terms of the normal distribution. Mann–Whitney *U* test was used to compare the means of tooth EtG concentrations in MAST point and teeth. Relations between continuous variables were examined with Pearson's correlation coefficient. All statistical data for $p < 0.05$ was considered significant. A probability of $p < 0.001$ was considered to be significant.

3. Results and discussion

Ages of the participants included in the study ranged between 35 and 60 years with a mean of 43.34 ± 6.83 years. All participants were male. According to the MAST outcomes, 14 of the patients were non-hazardous alcohol users, and 9 were alcohol abusers, while 6 patients verbally declared that they were abstainers. On the analyses, EtG levels was found to be ranged between EtG < LOD and 23.39 pg/mg in dental tissues. EtG was observed as <LOD in dental

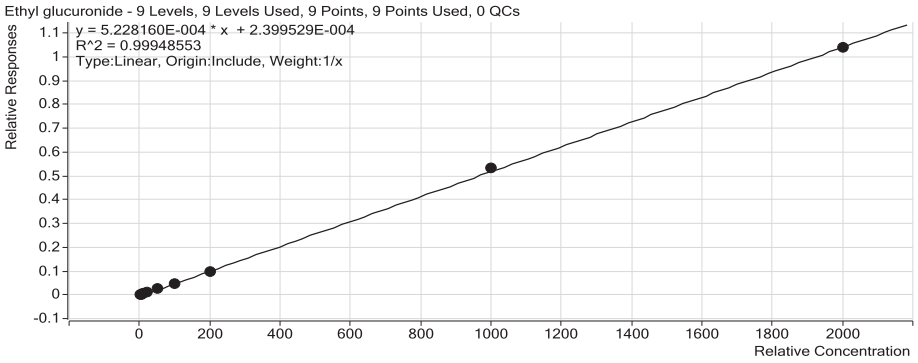


Fig. 2. Calibration curve for standard solutions.

specimens of 6 abstainers (Table 2). Calibration curve of the standard samples is shown in Fig. 2. Graphs of the analyses are presented in Fig. 3.

A significant correlation was observed between EtG values measured in the dental tissue and MAST outcomes of the statistical analyses ($r = 0.914$) (Fig. 4).

In this study, we demonstrated that measurement of EtG in dental tissues could be used to determine alcohol intake behavior. Existence of the statistical correlation between EtG values measured in the dental tissue and MAST outcomes suggested that EtG could be used in forensic toxicological investigations.

Using of the body fluids may be impossible, especially in postmortem forensic toxicological investigations, in cases of the significant deterioration of the body integrity as in postmortem decomposition and severe traumatic injuries, etc.[18] In these situations, durable tissues are needed for analyses.[19,20] Tooth is a durable tissue due to its compact structure.[13,14] Because of bacterial activity, toxicological analyses sometimes may yield false-positive or -negative results, which making difficult to interpret the results. Therefore, the least influenced tissues by bacterial contamination are recommended to be utilized in postmortem investigations. We believe the dental tissue is a good alternative sample for this thanks to its compact structure and its rich blood vessel network.

In the studies subjecting living cases, duration required for detection of EtG was stated to differ.[12] In a study investigating postmortem body fluids, time for detection of EtG was sorted as intraocular fluid > urine > blood.[6] This duration is longer in keratinous tissues.[10,11] Dental tissue suggested could be used in revealing of the alcohol intake behavior in longer term.

Significant results were obtained in the studies examining the correlation between alcohol intake behavior and EtG levels using blood, nails and hair samples.[21–23] In our study; a significant correlation was found between EtG levels and MAST outcomes

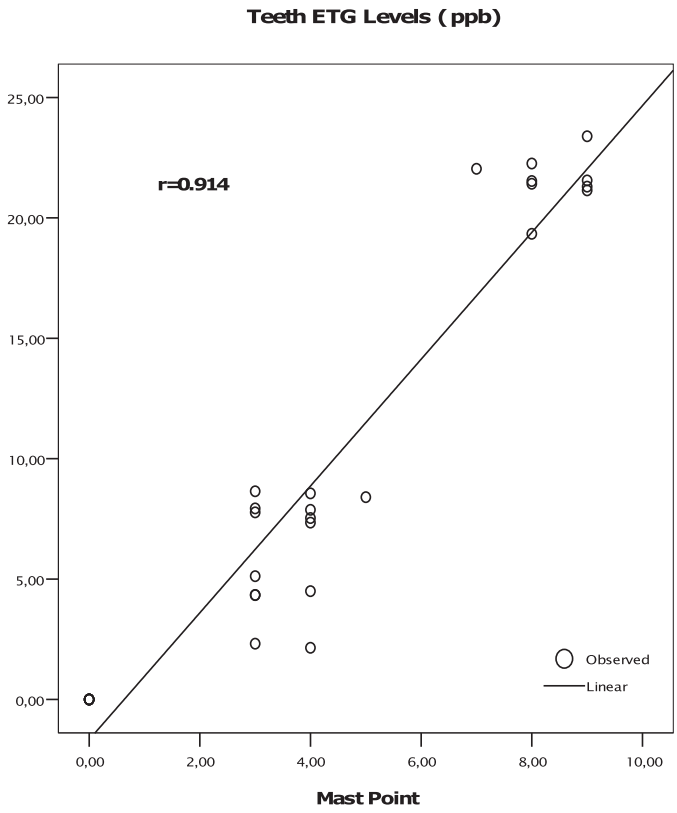
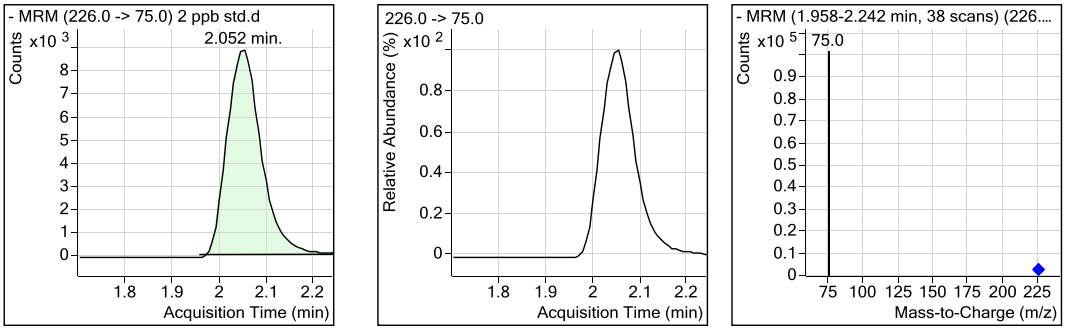


Fig. 4. Demonstrating of the correlation between EtG and MAST outcomes.

ISTD Compound *: Ethyl glucuronide D5.*



Target Compound *: Ethyl glucuronide.*

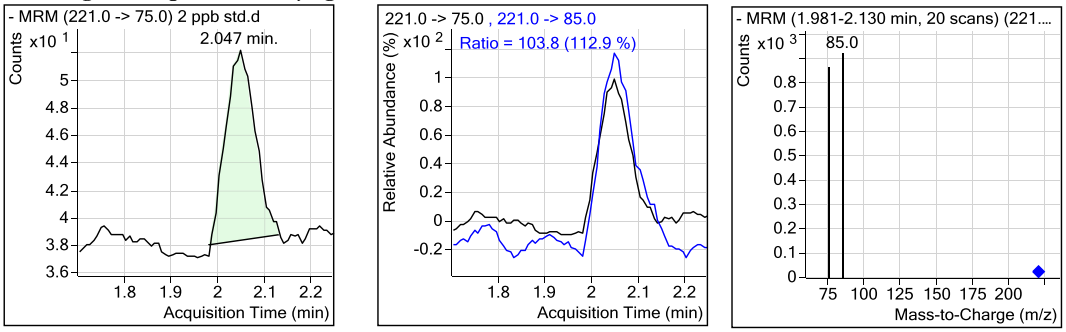


Fig. 3. Compound graphics.

($r = 0.914$). Similarly, there are several studies in the literature suggesting EtG level as an indicator of the alcohol intake behavior in postmortem cases.[6]

It is stated in the literature that demonstration of EtG after a certain period of time is impossible. EtG is superior to ethanol, although the demonstration of it in the body fluids like blood and urine is possible only within certain time limits.[12] Thus, keratinous tissues such as hair and nails might be needed to allow assessment for longer periods.[23–25] In their study, Jones et al. analyzed EtG in hair and nail samples and emphasized that nail samples could be used in revealing of the alcohol intake behavior.[26] Successful results were achieved also in a study about the demonstration of EtG in bone marrow, which is also a protected like dental tissue.[9]

In the studies regarding EtG analyses, GC/MS, LC/MS and LC–MS/MS devices have been used.[6,12,27,28] In the present study; we used LC/MS/MS. Various solutions were utilized in order to achieve the best extraction method. Water was used as a solvent in the extraction separately with acetonitrile, methanol and mixture of 80% acetonitrile–water and 50% acetonitrile–water. After the extractions, the peaks were examined. The best result for tooth samples was decided to come from extractions made with the mixture of 50% acetonitrile–water.

4. Conclusion

The findings of the present study demonstrated that dental tissue could be used for revealing of alcohol intake. There was a correlation between EtG levels detected in the dental tissue and alcohol intake behavior, suggesting that dental tissue could be used for alcohol intake behavior in the longer periods. Especially in postmortem forensic medical investigations, obtaining of dental tissue is always possible compared to blood, urine and intraocular fluid. Dental tissue can be used in the cases of the deterioration of bodily integrity as in decomposed or severely injured cases. However, further studies are needed to reveal the conditions in which EtG yields false-positive results. Particularly studies dealing with cases suffered from diseases causing tooth ischemia, and clarifying effects of use of toothpaste and mouthwash solutions on EtG levels are needed.

Ethical Approval

None declared.

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None declared.

Contributorship

All authors included in the authors list have contributed to the data collection, data evaluation, writing of manuscript, language use etc. And no professionals other than authors contributed to the any process of during article preparation.

Conflict of Interests

There are no Conflict of interests for any of authors.

References

1. World Health Organization. *Global status report on alcohol and health*. Geneva: World Health Organization; 20119–12.
2. O'Neal CL, Poklis A. Postmortem production of ethanol and factors that influence interpretation: a critical review. *Am J Forensic Med Pathol* 1996;**17**: 8–20.
3. Corry JEL. A review. Possible sources of ethanol ante- and postmortem: its relationship to the biochemistry and microbiology of decomposition. *J Appl Bacteriol* 1978;**44**:1–56.
4. Pounder D. Dead sober or dead drunk? May be hard to determine. *BMJ* 1998;**316**:87.
5. Geerrtinger P, Bodenhoff J, Helweg-Larsen K, Lund A. Endogenous alcohol production by intestinal fermentation in sudden infant death. *Z Rechtsmed* 1982;**89**:167–72.
6. Keten A, Tümer AR, Balseven Odabasi A. Measurement of ethyl glucuronide in vitreous humor with liquid chromatography-mass spectrometry. *Forensic Sci Int* 2009;**193**:101–5.
7. Foti RS, Fisher MB. Assessment of UDP-glucuronosyl transferase catalyzed formation of ethyl glucuronide in human liver microsomes and recombinant UGTs. *Forensic Sci Int* 2005;**153**:109–16.
8. Schmitt G, Aderjan R, Keller T, Wu M. Ethyl glucuronide: an unusual ethanol metabolite in humans. Synthesis, analytical data, and determination in serum and urine. *J Anal Toxicol* 1995;**19**:91–4.
9. Schloegl H, Rost T, Schmidt W, Wurst FM, Weinmann W. Distribution of ethyl glucuronide in rib bone marrow, other tissues and body liquids as proof of alcohol consumption before death. *Forensic Sci Int* 2006;**156**:213–8.
10. Kerekes I, Yegles M, Grimm U, Wennig R. Ethyl glucuronide determination: head hair versus non-head hair. *Alcohol Alcohol* 2009;**44**:62–6.
11. Kintz P, Villain M, Vallet E, Etter M, Salquebre G, Cirimele V. Ethyl glucuronide: unusual distribution between head hair and pubic hair. *Forensic Sci Int* 2008;**176**:87–90.
12. Høiseth G, Bernard JP, Karinen R, Johnsen L, Helander A, Christophersen AS, et al. A pharmacokinetic study of ethyl glucuronide in blood and urine: applications to forensic toxicology. *Forensic Sci Int* 2007;**172**:119–24.
13. Nuzzolese E, Di Vella G. Future project concerning mass disaster management: a forensic odontology prospectus. *Int. Dent J* 2007;**572**:261–6.
14. Iijima T, Zhang JQ. Three-dimensional wall structure in the innervation of dental pulp blood vessels. *Microsc Res Tech* 2002;**56**:32–41.
15. Matthews B, Andrew D. Microvascular architecture and exchange in teeth. *Microcirculation* 1995;**2**:305–13.
16. Yoshida S, Ohshima H. Distribution and organization of peripheral capillaries in dental pulp and their relationship to odontoblasts. *Anat Rec* 1996;**245**:313–26.
17. Olaisen B, Stenersen M, Mevåg B. Identification by DNA analysis of the victims of the August 1996 Spitsbergen civil aircraft disaster. *Nat Genet* 1997;**15**:402–5.
18. Brinkmann B, Madea B. *Handbuch gerichtliche Medizin*, vol. 1. Berlin, Heidelberg, New York: Springer; 2004150–65.
19. Skopp G. Preanalytic aspects in postmortem toxicology. *Forensic Sci Int* 2004;**142**:75–100.
20. Levisky JA, Bowerman DL, Jenkins WW, Johnson DG, Karch SB. Drugs in postmortem adipose tissues: evidence of antemortem deposition. *Forensic Sci Int* 2001;**121**:157–60.
21. Morini L, Colucci M, Ruberto MG, Groppi A. Determination of ethyl glucuronide in nails by liquid chromatography tandem mass spectrometry as a potential new biomarker for chronic alcohol abuse and binge drinking behavior. *Anal Bioanal Chem* 2012;**402**:1865–70.
22. Neumann T, Helander A, Dahl H, Holzmann T, Neuner B, Gerlach1 EW, et al. Value of ethyl glucuronide in plasma as a biomarker for recent alcohol consumption in the emergency room. *Alcohol Alcohol* 2008;**43**:431–5.
23. Wurst MF, Skipper GE, Weinmann W. Ethyl glucuronide-the direct ethanol metabolite on the threshold from science to routine use. *Addiction* 2003;**98**: 51–6.
24. Politi L, Zucchella A, Morini L, Stramesi C, Polettini A. Markers of chronic alcohol use in hair: comparison of ethyl glucuronide and cocaethylene in cocaine users. *Forensic Sci Int* 2007;**172**:23–7.
25. Morini L, Politi L, Polettini A. Ethyl glucuronide in hair. A sensitive and specific marker of chronic heavy drinking. *Addiction* 2009;**104**:915–20.
26. Jones J, Jones M, Plate C, Lewis D, Fendrich M, Berger L, et al. Liquid chromatography-tandem mass spectrometry assay to detect ethyl glucuronide in human fingernail: comparison to hair and gender differences. *Am J Anal Chem* 2012;**3**:83–91.
27. Agius R, Nadulski T, Kahl HG, Schräder J, Dufaux B, Yegles M, et al. Validation of a headspace solid-phase microextraction-GC-MS/MS for the determination of ethyl glucuronide in hair according to forensic guidelines. *Forensic Sci Int* 2010;**196**:3–9.
28. Helander A, Kenan N, Beck O. Comparison of analytical approaches for liquid chromatography/mass spectrometry determination of the alcohol biomarker ethyl glucuronide in urine. *Rapid Commun Mass Spectrom* 2010;**24**:1737–43.